genome represented in the synthetic target. Oligonucleotide 11451 anneals to oligonucleotide 11454. Oligonucleotide 11450 anneals to oligo 11453.

The following solutions were assembled in triplicate and nanopure water added to a final volume of 20 μL.

Solution	0.3 ng Target	1 μg Probe	rlu
1.	+	11451	391
2.	+	11450	241
3.	+	none	28
4.	-	11451	248
5.	-	11450	30
6.	-	none	24

The assembled solutions were incubated at 92°C for 5 minutes, then cooled at room temperature for 10 minutes. Master mix was prepared as in Example 1 using 10 units Klenow exo- polymerase and 4 units NDPK. Twenty microliters of master mix were added to each tube and incubated at 37°C for 15 minutes. Five microliters of each solution were then combined with 100 μL of L/L reagent (Promega F202A) and light output measured immediately on a Turner® TD20/20 luminometer. The average relative light units (rlu) are recorded in the table above

Using each of the interrogation probes with the target appears to give strong net signal. The top probe (11451) however, gives very strong signal

alone, possibly due to hairpin formation, and is less suitable for interrogation. The bottom interrogation probe (11450) is the better for interrogation.

11453
5'CTTGAAGCATAGTTCTTGTTTTTAAACTTTGTCCATCTTGAGCCGCTTGA
GTTGCCTTAGTTTTAATAGT 3' SEQ ID NO:31

11454
5'ACTATTAAAACTAAGGCAACTCAAGCGGCTCAAGATGGACAAAGTTTA
AAAACAAGAACTATGCTTCAAG 3' SEQ ID NO:33

11451
5' AGTTCTTGTTTTTAAACTTTGTCCATCTTG 3' SEQ ID NO:32

11450
5' CAAGATGGACAAAGTTTAAAAACAAGAACT 3' SEQ ID NO:34

Example 12: Interrogation For Loss of Heterozygosity - CMV

The use of an interrogation assay to determine loss of heterozygosity with a synthetic cytomegalovirus (CMV) target is demonstrated in this example.

The CMV target was chosen because the interrogating probe oligonucleotides 9211 (SEQ ID NO:35) and 9212 (SEQ ID NO:36) had been previously used and well characterized. Oligonucleotides 10800 (SEQ ID NO:37) and 10801 (SEQ ID NO:38) were annealed to produce a synthetic target, "A", representing a fragment of the CMV genome. Likewise,

oligonucleotides 10803 (SEQ ID NO:39) and 10805 (SEQ ID NO:40) were annealed to produce a synthetic target, "G" representing a fragment of the CMV genome. Targets A and G are identical except at one nucleotide position where they have the nucleotide resulting in their name. Both targets have SacI overhangs.

The targets were cloned into the SacI restriction site of pZERO-2 plasmid (Invitrogen) and transformed into TOP10 *E. coli* cells (Invitrogen). The presence of the correct nucleotide sequence in the A and G clones was confirmed by sequencing. However, the G clone was found to contain an unintended mutation at the nucleotide position three bases in from the 5′ end of the region that anneals to the interrogation probes. Because this mismatch is near the 5′ end of the interrogation probe annealing sequence, it should not affect the interrogation results.

The following five target solutions were created with the A and G clones:

1. Hetero: 125 pg A and 125 pg G/microliter
2. LOH A: 125 pg A and no G/microliter
3. LOH G: no A and 125 pg G/microliter
4. Mix Ag: 125 pg A and 62 pg G/microliter
5. Mix Ga: 62 pg A and 125 pg G/microliter

These target solutions were PCR amplified with the JH67 (SEQ ID NO:41) and 11077 (SEQ ID NO: 42) probes in the following reaction:

2 µL Target solution

1 µL Probes JH67 and 11077 (50 pmol each)

1 µL 10 mM dNTPs

5 µL 10X Taq buffer

1 µL Taq DNA polymerase

40 µL water

The PCR cycling parameters were: 96°C, 1 minute; (94°C, 15 seconds; 60°C, 30 seconds; 72°C, 45 seconds) x 15; 72°C, 45 seconds. The entire PCR reaction was then purified with 500 µL Wizard™ PCR purification resin (Promega, A7170) according to manufacturer's instructions. The DNA was eluted with 30 µL TE buffer. A standard interrogation reaction with 6 µL target and 1 µg interrogation probe, was performed with the exception that 2 units of Klenow exo- were used per reaction. Four microliters of the final reaction were combined with 100 µL of L/L reagent and the relative light units measured.

	Heterozygote		LOH A		LOH G		Mix Ag		Mix Ga		Oligo Alone
No oligo	30	40	65	29	34	51	19	59	26	41	-
A oligo	279	340	74	329	27	27	258	309	50	164	5.2
	308	372	76	339	20	26	351	330	83	167	5.2
G oligo	302	324	37	91	285	272	127	106	245	302	6.3
	278	325	30	87	256	187	113	124	215	357	6.3
A:G ratio	1.01	1.10	2.26	3.76	0.09	0.11	2.54	2.78	0.29	0.50	

G:A ratio	0.99	0.91	0.44	0.27	11.59	8.71	0.39	0.36	3.46	1.99

These data illustrate that LOH can be determined using this method with appropriate interrogation probes.

10800 5′ CGTGTATGCCACTTTGATATTACACCCATGAACGTG CTCATCGACGTGAACCCGCACAACGAGCT 3′ SEQ ID NO:37

10801 5′ CGTTGTGCGGGTTCACGTCGATGAGCACGTTCATGG GTGTAATATCAAAGTGGCATACACGAGCT 3′ SEQ ID NO:38

10803 5′ CGTGTATGCCACTTTGATATTACACCCGTGAACGTG CTCATCGACGTGAACCCGCACAACGAGCT 3′ SEQ ID NO:39

10805 5′ CGTTGTGCGGGTTCACGTCGATGAGCACGTTCACGG GTGTAATATCAAAGTGGCATACACGAGCT 3′ SEQ ID NO:40

JH67 5' TCACACAGGAAACAGCTATGACCATG 3' SEQ ID NO:41

11077 5′ GCAAGGCGATTAAGTTGGGTAACG 3′ (M13 forward probe) SEQ ID NO:42

9211 5′ CACTTTGATATTACACCCATG 3′ SEQ ID NO:35

9212 5′ CACTTTGATATTACACCCGTG 3′ SEQ ID NO:36

Example 13: <u>Determination of Viral Load</u>

This example illustrates that the presence of viral nucleic acid in serum samples can be determined to a detection level of ten copies of viral nucleic acid per sample.

Hepatitis C Virus (HCV) RNA was isolated from infected or uninfected human serum samples. A two-step RT-PCR was performed using HCV-specific probes and about 1000 viral equivalents of RNA, and samples were interrogated using the interrogation probe HCV1 (SEQ ID NO:43).

Two HCV positive samples, one HCV negative sample, and a water control were analyzed in duplicate. The interrogation reaction was added to 100 µL of L/L reagent (Promega F202A) and the light output measured immediately on a Turner® TD20/20 luminometer. The average relative light unit values were as follows.

Water control	38.6
HCV minus	239.0
HCV positive (1)	1261.0
HCV positive (2)	1390.0

To determine the sensitivity of viral detection using this technology, RT-PCR was performed on HCV positive and HCV negative controls as well as samples estimated to contain 1000, 100, and 10 viral RNA copies. Twenty five microliters of each amplification reaction were purified using magnetic silica as follows, and eluted in 100 µL water.

1. 200 μL of a slurry containing 15 μL MagneSil™ paramagnetic particles (Promega) in solution containing 0.4 M guanidine thiocyanate and 0.08 M potassium acetate were added to each sample.
2. The MagneSil™ paramagnetic particles were mixed in the solutions and held against the side of the tube with a magnet.
3. The particles were washed twice with 200 μL of 70% ethanol by addition of the solution to the tubes, resuspension of the particles in the solution, recapture of the particles against the tube walls with the magnet and removal of the particle-free solution.
4. The particles were resuspended in fifty microliters of water.
5. 200 μL 0.4 M GTC and 0.08 M potassium acetate were added to each.
6. Step 2 was repeated as described above except that three washes with 70% ethanol were performed.
7. The particles were resuspended in 100 μL water, the particles were captured against the side of the tube, and the solution containing the purified DNA was transferred to a new tube.

Four microliters of the eluted DNA were interrogated using 1 microliter of the interrogation probe diluted to a total of twenty microliters with water. The nucleic acid solutions were heated to 95°C for three

minutes, then placed in a 37°C incubator for 10 minutes. The following master mix was assembled:

10X DNA Polymerase Buffer (Promega M195)	20 μL
40 mM Sodium Pyrophosphate (Promega C113)	5 μL
10U/μl Klenow Exo Minus (Promega M218)	5 μL
NDPK (Sigma, NO379 at 10 U/μL in water)	1 μL
ADP (Sigma A5285, 10 μM in water)	2 μL
Water	67 μL
	100 μL

Twenty microliters of master mix were added to each of the heated nucleotide mixes after incubation at 37°C for 10 minutes. The resulting reactions were incubated for 15 minutes at 37°C and then added to 100 μL L/L reagent (Promega, F202A) and the light produced was immediately read using a Turner® TD20/20 luminometer. The interrogation reaction was added to 100 μL of L/L reagent and the light output measured on a Turner® TD 20/20 luminometer. Ten copies of HCV are readily detected in this assay. The average relative light unit (rlu) values were as follows.

Sample	rlu
Water	49.0
Water	54.2
HCV neg control	59.4
HCV neg control	62.1
HCV pos control	653.7
HCV pos control	743.1
HCV 1000 copies	460.7

HCV 1000 copies	429.5
HCV 100 copies	405.1
HCV 100 copies	404.3
HCV 10 copies	184.9
HCV 10 copies	179.5

HCV1:

5' CTGCTAGCCGAGTAGTGTTGGGTCGCGAAAGGCCTTGTGG 3'

SEQ ID NO:43

Example 14: Interrogation of DNA Sequences from Genetically Modified Organisms

According to European Union (EU) Regulation on Novel Foods and Novel Food Ingredients, adopted in 1997, genetically modified foods must be labeled as such if they are "no longer equivalent" to their conventional counterparts. This includes when the foods have a different composition, use or nutritional value from the conventional food. The EU subsequently decided that the presence of just a fragment of genetically modified protein or DNA is enough to make the product "no longer equivalent" to conventional products for soya and maize and, therefore, such products require labeling.

Genetically modified organisms (GMO), particularly plants, are often genetically modified to include the exogenous specific DNA of interest along with an exogenous transcription sequence such as the 35S promoter and the NOS terminator. In this example, the DNA of soya and maize samples are analyzed for the presence or absence of the 35S

promoter and NOS terminator. The PCR Primers 35S-1 (SEQ ID NO:44) and 35S-2 (SEQ ID NO:45) were used to prepare a 235 bp PCR product. The Primers NOS-1 (SEQ ID NO:46) and NOS-2 (SEQ ID NO:47) were used to prepare a 220bp PCR product.

GMO positive and negative control DNA (20 ng) were PCR amplified using 50 pmol of the 35S promoter and NOS terminator PCR primer pairs. The PCR cycling profile was 94°C, 3 minutes; (94°C, 30 seconds; 54°C, 40 seconds; 72°C, 1 minute) x 40; 72°C, 3 minutes. The resulting PCR products (25 µL) were purified using magnetic silica and eluted in 100 µL water as described in Example 13. Four microliters of the eluted PCR products were used in a standard interrogation assay as described in Example 13 and the relative light unit (rlu) results are detailed in the following table. The 35S interrogation probes used were 11211 (SEQ ID NO:48) and 11210 (SEQ ID NO:49). The NOS interrogation probes used were 11212 (SEQ ID NO:50) and 11213 (SEQ ID NO:51).

DNA	PCR Oligos	Interrogation Oligos	rlu
GMO minus, soy	35S	11210	166.6
GMO minus, soy	35S	11210	172.0
GMO minus, soy	35S	11211	206.8
GMO minus, soy	35S	11211	205.8
GMO minus, soy	35S	none	95.7
GMO minus, maize	35S	11210	245.0

GMO minus, maize	35S	11210	254.3
GMO minus, maize	35S	11211	271.3
GMO minus, maize	35S	11211	275.7
GMO minus, maize	35S	none	116.0
GMO positive, soy	35S	11210	1456.0
GMO positive, soy	35S	11210	1442.0
GMO positive, soy	35S	11211	1546.0
GMO positive, soy	35S	11211	1529.0
GMO positive, soy	35S	none	865.0
GMO positive, maize	35S	11210	1252.0
GMO positive, maize	35S	11210	1299.0
GMO positive, maize	35S	11211	1358.0
GMO positive, maize	35S	11211	1361.0
GMO positive, maize	35S	none	705.6
GMO minus, soy	NOS	11212	73.9
GMO minus, soy	NOS	11213	75.8
GMO minus, soy	NOS	none	76.1
GMO positive, soy	NOS	11212	615.0
GMO positive, soy	NOS	11213	616.6
GMO positive, soy	NOS	none	98.0

The above data demonstrate that the interrogation reaction works for the identification of presence or absence of GMO DNA in DNA samples isolated from soy and maize products. The 35S PCR product gave high background values by itself, which can be reduced by using a primer with phosphorothioate linkages near the 5'-terminus for

the PCR reaction followed by exo6 treatment to remove one strand of the PCR product as described in Example 13 and below. The PCR primers 35S1 and NOS1 were resynthesized to have phosphorothioate linkages between the first five bases at the 5′ end. The PCR reaction was repeated and the resulting PCR product treated with Exo6 and purified as described in Example 13.

Four microliters of the purified DNA were used for the standard interrogation assay using the NOS primer 11212 and the 35S primer 11211 with 5 units of Klenow exo-. The rlu data obtained are in the table below.

DNA	PCR oligos	Interrogation oligo	rlu
GMO minus, soy	NOS	11212	52.3
GMO minus, soy	NOS	11211	60.2
GMO minus, soy	NOS	none	53.3
GMO positive, soy	NOS	11212	277.1
GMO positive, soy	NOS	11211	84.4
GMO positive, soy	NOS	none	75.7
GMO minus, soy	35S	11212	57.8
GMO minus, soy	35S	11211	66.9
GMO minus, soy	35S	none	54.6
GMO positive, soy	35S	11212	99.7
GMO positive, soy	35S	11211	397.6
GMO positive, soy	35S	none	86.0

GMO positive, soy	35S + NOS	11212	249.4
GMO positive, soy	35S + NOS	11211	290.1
GMO positive, soy	35S + NOS	11211 + 11212	482.5
GMO positive, soy	35S + NOS	none	70.5

This method greatly reduced the background from the 35S PCR product and permitted better discrimination between the GMO positive and GMO minus DNA samples. Also, this example again demonstrates the utility of the technology for multiplexing both the PCR reaction and the interrogation reaction. As seen in the last four reactions above, the data show that the use of multiple PCR probes and/or multiple interrogation probes leads to identification of GMO organisms.

35S promoter PCR primers:

35S-1	5' GATAGTGGGATTGTGCGTCA 3'	SEQ ID NO:44
35S-2	5' GCTCCTACAAATGCCATCA 3'	SEQ ID NO:45

NOS terminator PCR primers

NOS-1	5' TTATCCTAGTTTGCGCGCTA 3'	SEQ ID NO:46
NOS-2	5' GAATCCTGCTGCCGGTCTTG 3'	SEQ ID NO:47

35S Interrogation oligonucleotide probes:

11211	5' GCAAGTGGATTGATG 3'	SEQ ID NO:48
11210	5' CCAACCACGTCTTCAAA 3'	SEQ ID NO:49

NOS Interrogation oligonucleotide probes

11212	5' TTTATGAGATGGGTTT 3'	SEQ ID NO:50

11213 5′ ATGATTAGAGTCCCG 3′ SEQ ID NO:51

Example 15: HPLC Separation of dNTPs After Interrogation Assay, but Prior to Phosphate Transfer and Light Production

Large-volume pyrophosphorylation assays were performed on matched and mismatched probe/target hybrids. The released nucleotides were separated by high performance liquid chromatography (HPLC) and their fractions collected. NDPK terminal phosphate transfer reactions were performed on these concentrated fractions and luciferase assays conducted to illustrate discrimination between the original matched and mismatched hybrid treated samples.

Target/probe hybrids were formed by combining 315 ng of the synthetic wild type CMV target oligonucleotide with either 10.5 μg wild type CMV probe for a matched hybrid, or 10.5 μg mutant CMV probe for a mismatched hybrid, and adding water to a final volume of 200 μL. The oligonucleotides were CV 12 (SEQ ID NO:2), CV 15 (SEQ ID NO:5), and CV 16 (SEQ ID NO:6), as previously described in Example 1. These solutions were heated to 95°C for at least 5 minutes, then cooled at room temperature for at least 10 minutes.

The following master mix was prepared.

337.5 μL Nanopure water (Promega, AA399)

90.0 μL 10X DNA Polymerase buffer (Promega,

M195A)

11.25 µL 40 mM NaPPi (Promega, C113)

Master mix (210 µL) was added to each of the above hybrid solutions and 5.8 units of Klenow exo- (Promega, M218A) were added to each. The solutions were then incubated at 37°C for 15 minutes and stored on ice. HPLC separation of the dNTPs was performed.

HPLC separation of dATP, dCTP, dGTP and TTP was performed on a 100 X 4.6 mm, 3 µ Luna C18 column [Perrone and Brown, *J. Chromatography*, **317**:301-310 (1984)] from Phenomenex. The column was eluted with a linear gradient of 97 percent buffer A (100 mM triethylammonium acetate, pH 7) to 92 percent buffer A over a period of 12 minutes. The composition of buffer B is 80:20 acetonitrile:35 mM triethylammonium acetate. Detection was monitored by absorbance at 250, 260 and 280 nm. Under these conditions, dCTP was found to elute between 4 and 4.5 minutes, TTP and dGTP eluted as two peaks between 7 and 7.5 minutes, and dATP eluted from 9 to 9.5 minutes.

The fractions containing the free dNTPs were collected and lyophilized. Fraction one contained dCTP, fraction two contained dGTP and TTP, and fraction three contained dATP.

Each fraction was reconstituted in 100 µL of nanopure water. Ten microliters of each fraction, or 10 µL of water as a control, were added to a 10 µL mixture of water, 10X DNA Polymerase Buffer, and ADP

so that the final concentration was 1X DNA pol buffer and 0.1 μM ADP. NDPK (0.005 units) was added to each tube in one set of the tubes and an equal amount of water was added to each tube in the other set of tubes. Samples and controls were incubated at 37°C for 15 minutes, 10 μL added to 100 μL of L/L reagent and the light output was measured on a Turner® TD10/20 luminometer. The relative light units (rlu) results obtained are shown below:

Sample	Trial 1	Trial 2	Trial 3	Avg rlu
Matched hybrid with NDPK				
Fraction 1	206.6	200.6	205.9	204.4
Fraction 2	839.4	851.6	833.9	841.6
Fraction 3	1149.0	1150.0	1169.0	1156
Mismatched hybrid with NDPK				
Fraction 1	101.8	97.0	98.9	99.9
Fraction 2	386.1	387.3	382.2	385.2
Fraction 3	412.4	409.9	416.5	412.9
Match hybrid without NDPK				
Fraction 1	6.8	6.5	--	6.6
Fraction 2	10.9	11.5	--	11.2
Fraction 3	33.0	37.8	--	35.4
Mismatched hybrid without NDPK				
Fraction 1	6.2	6.7	--	6.4
Fraction 2	8.3	8.4	--	8.4
Fraction 3	13.4	13.5	--	13.4
No dNTP	7.9	7.5	--	7.7

As is seen from the above data, the fraction one match:mismatch ratio is 2.1, fraction 2 match:mismatch ratio is 2.2 and fraction 3 match:mismatch ratio is 2.8. The data therefore

demonstrate the utility of using HPLC separation of individual nucleotides followed by NDPK conversion to ATP, the preferred substrate of luciferase. Fraction 3 provides a slightly higher match:mismatch ratio owing to the presence of dATP in the nucleotide HPLC fraction. Nevertheless, HPLC separation of identifier nucleotides is useful in the interrogation assays of the present invention.

CV12
5′CCAACAGACGCTCCACGTTCTTTCTGACGTATTCGTGCAGCATGGTCTGCG
AGCATTCGTGGTAGAAGCGAGCT 3′ SEQ ID NO:2

CV15 5′ CTACCACGAATGCTCGCAGAC 3′ SEQ ID NO:5

CV16 5′ CTACCACGAATGCTCGCAGAT 3′ SEQ ID NO:6

Example 16: Mass Spectrometry for <u>Nucleotide Detection</u>

The mass spectrometer uses the ratio of molecular mass to charge of various molecules to identify them. Nucleic acids are made up of four different base molecules, each with a different mass to charge ratio. In this example, the capability to use mass spectrometry for separation of the nucleotides that make up DNA is demonstrated.

The ESIMS (Electro Spray Ion Mass spectrometry) spectra of 1 μM and 0.1 μM NTP molecules were determined (Fisons Instruments, VG Platform). The samples were prepared by diluting 1:1

with acetonitrile/water/1% NH_4OH. A 20 μL injection was made for each sample. Therefore, 10 picomoles of each dNTP are in the 1 μM sample injection, and 1 picomole of each dNTP is in the 0.1 μM sample injection.

Each of the dNTPs is observed in the 1 μM sample along with the dNTP+Na^+ peaks. There was a 485 peak also present, which is an impurity in the system or samples. The peaks for each of the dNTPs are significantly diminished in the 0.1 μM sample; only the dATP peak is above the noise level. Therefore, the difference between the 1 and 0.1 μM samples can be qualitatively determined, which indicates the ability to determine the difference between interrogation samples in which the probe and target are matched and mismatched at the 3'-terminal region of the probe.

Example 17: Detection of Human Immunodeficiency <u>Virus (HIV) Drug-Resistant Mutants</u>

Chemotherapeutic selection pressure *in vivo* often results in mutations within the genome of the infectious agent that the drug is intended to destroy. This demonstration of evolutionary adaptation is widely reported for human immunodeficiency virus (HIV) under the selective pressure of protease inhibitors or reverse transcriptase inhibitors (Martinez-Picado, *J. Virology*, **73**:3744-3752, 1999; Back, *EMBO J.*, **15**:4040-4049, 1996).

The first viral mutants to be selected during therapy are typically those with single-amino-acid substitutions. Some of the nucleotides of the HIV reverse transcriptase (RT) and protease genes are known in the art to be "hotspots" for developing such point mutations. Additional mutations accumulate with ongoing therapy. After about 6 months to 1 year of treatment with AZT, HIV typically mutates the RT gene and so becomes resistant to treatment.

The ability to detect and identify such viral mutant genomes in a reliable and sensitive assay would assist with understanding the progression of the infection and with developing the best treatment regimens for infected individuals. Switching to a different treatment course before or as soon as a resistant mutant virus takes hold is important in prolonging patient life.

This example demonstrates that drug resistant mutations that occur within the HIV-1 reverse transcriptase gene, when under the selective pressure of reverse transcriptase inhibitors, such as the nucleoside analog drugs AZT and ddI, can be detected using the process of the invention. Three specific "hotspot" sites of RT mutation were chosen for study. These three mutations all exist within a short region of the RT gene, spanning about 10 amino acids, from codon 65 to 75 of the protein.

Codon 67 (Site 1) of RT changes from GAC to AAC in the presence of the drug AZT, codon 70 (Site 2) changes from AAA to AGA in the presence of AZT, and codon 75 (Site 3) changes from GTA to ATA in the

presence of the combination of drugs AZT and ddI. Target oligonucleotides were synthesized to span codons 65 through 81 of the RT genome of HIV-1 strain HXB2 wild type genome as well as oligonucleotides that vary only at one position as defined above for Site 1, Site 2, and Site 3 point mutations. Probe oligonucleotides exactly complementary to the wild type target and to the mutant targets at these three sites were also synthesized. The sequence and names of these oligonucleotides are listed below.

The probe oligonucleotides were dissolved in TE Buffer to a final concentration of 0.5 μg/μL. The target oligonucleotides were dissolved in TE Buffer to a final concentration of 5 μg/mL. One microliter of target was combined with 1 μL of probe and 18 μL of water; and for the controls, 1 μL of each oligonucleotide was combined with 19 μL of water. These solutions were then heated at 95°C for 3 minutes and cooled at room temperature for 10 minutes. Twenty microliters of master mix were then added to each tube. The master mix is described below.

Master Mix:

10X DNA Polymerase buffer (Promega, M195A)	120 μL
40 mM Sodium pyrophosphate	15 μL
Klenow exo- enzyme (1 U/μL; Promega, M218A)	15 μL
NDPK (1 U/μL)	6 μL
ADP (10 μM)	12 μL
Nanopure water	432 μL

The tubes with the master mix added were then incubated for 15 minutes at 37°C. Five microliters of the solutions were then combined with L/L reagent (Promega, F202A) and the light output was measured on a Turner® TD20/20 luminometer. The relative light unit (rlu) data obtained are listed below.

Solution	Target	Probe	Reading 1	Reading 2	Reading 3
1)	11814 (wt*)	--	2.55	3.82	10.78
2)	11815 (mut1*)	--	2.54	2.57	2.99
3)	--	11808 (wt1)	162.8	207.2	165.5
4)	--	11809 (mut1)	2.81	2.17	2.20
5)	11816 (mut2)	--	3.84	3.98	3.81
6)	--	11810 (wt2)	4.57	4.77	5.29
7)	--	11811 (mut2)	3.84	3.98	3.81
8)	11817 (mut3)	--	2.04	1.64	1.44
9)	--	11812 (wt3)	2.36	2.57	2.41
10)	--	11813 (mut3)	4.05	2.06	1.77
11)	11814 (wt)	11808 (wt1)	418.7	711.6	682.1
12)	11814	11809	20.69	29.05	21.25

	(wt)	(mut1)			
13)	11815	11808	218.4	185.6	118.1
	(mut1)	(wt1)			
14)	11815	11809	682.6	737.8	599.7
	(mut1)	(mut1)			
15)	11814	11810	1055.0	920.2	744.7
	(wt)	(wt2)			
16)	11814	11811	175.3	188.1	171.1
	(wt)	(mut2)			
17)	11815	11810	136.9	121.0	114.4
	(mut2)	(wt2)			
18)	11815	11811	822.3	865.9	729.0
	(mut2)	(mut2)			
19)	11814	11812	31.49	33.22	43.83
	(wt)	(wt3)			
20)	11814	11813	2.55	3.79	2.49
	(wt)	(mut3)			
21)	11815	11812	5.26	6.00	6.33
	(mut2)	(wt3)			
22)	11815	11813	77.58	78.46	82.85
	(mut2)	(mut3)			
23)	no DNA		2.18	2.48	1.37

* wt = wild type; mut = mutant. wt and mut a, 2, and 3 are defined hereinafter.

All three HIV RT drug-resistance mutations were detectable with discrimination of mutant:wild type rlu ratios ranging from about 3 to about 7. Probe 11808, which is directed to site one and is completely complementary to wild type target, had high background values when tested alone. The other oligonucleotides all had acceptably low levels of background.

Target and Probe Sequences

11808 5' CCATTTAGTACTGTCT 3' SEQ ID NO:52
HIV WT Probe Site 1

11809 5' CCATTTAGTACTGTTT 3' SEQ ID NO:53
HIV Mutant Probe Site 1

11810 5' CTAGTTTTCTCCATTT 3' SEQ ID NO:54
HIV WT Probe Site 2

11811 5' CTAGTTTTCTCCATCT 3' SEQ ID NO:55
HIV Mutant Probe Site 2

11812 5' TTCTCTGAAATCTACT 3' SEQ ID NO:56
HIV WT Probe Site 3

11813 5' TTCTCTGAAATCTATT 3' SEQ ID NO:57
HIV Mutant Probe Site 3

11814 5' AAAAAAGACAGTACTAAATGGAGAAAACTAGTA
GATTTCAGAGAACTTAA 3' SEQ ID NO:58
HIV WT Target

11815 5' AAAAAAAACAGTACTAAATGGAGAAAACTAGTAGA
TTTCAGAGAACTTAA 3' SEQ ID NO:59
HIV Mutant Target Site 1

11816 AAAAAAGACAGTACTAGATGGAGAAAACTAGTAGATTTCAG
AGAACTTAA 3' SEQ ID NO:60
HIV Mutant Target Site 25'

11817 5'AAAAAAGACAGTACTAAATGGAGAAAACTAA
TAGATTTCAGAGAACTTAA 3' SEQ ID NO:61
HIV Mutant Target Site 3

Example 18: Detection of *E. coli* repetitive sequence <u>without nucleic acid amplification</u>

In this example repetitive sequence in *E. coli* is detected without the need for amplification of the target sequence prior to pyrophosphorylation. This target sequence is denoted as 'colirep'.

Oligonucleotide 11707 (SEQ ID NO:62) is totally complementary to a segment of colirep DNA sequence. Twelve microliters of oligonucleotide 11707 solution (1mg/mL) were combined with 204 μL of water to make solution A. Another solution was prepared by combining 4 μL of 11707 (1 mg/mL) with 204 μL water and 8 μL 10 mM Tris, pH 8.0 to make solution B. The *E. coli* is Sigma cat#D4889, *E. coli* Strain B ultra pure.

Four nanograms (2 μL) *E. coli* DNA were combined with 18 μL solution A and with 18 μL solution B in separate tubes. Similarly, 40 ng *E. coli* DNA was combined with 18 μL solution A and with 18 μL solution B in separate tubes. These solutions were then incubated at 92°C for 3 minutes and cooled at room temperature for 15 minutes. The following master mix was assembled:

10X DNA Polymerase buffer 240 μL

40 mM NaPPi	30 μL
Klenow exo- (10 U/μL)	30 μL
NDPK (1 U/μL)	12 μL
10 μM ADP (Sigma)	24 μL
water	864 μL

Twenty microliters of master mix were added to each reaction and they were then incubated at 37°C for 15 minutes. One hundred microliters of L/L Reagent were then added and the relative light output (rlu) immediately measured with a Turner® TD 20/20 luminometer. The rlu were:

Solution	rlu-1	rlu-2	rlu-3	Average
Tris	2.85	3.562	3.059	3.157
11707 (A)	13.69	12.13	13.67	13.16
11707 (B)	7.473	7.234	6.981	7.259
40 ng DNA+Tris	75.62	75.52	73.24	74.79
40 ng DNA + 11707(A)	97.71	134.2	105.1	112.3
40 ng DNA + 11707 (B)	81.46	87.56	76.28	81.77
4 ng DNA+Tris	6.719	8.084	5.882	6.895
4 ng DNA + 11707 (A)	24.50	25.97	25.17	25.21
4 ng DNA + 11707 (B)	15.69	17.22	16.99	16.63

The data reflect that oligonucleotide probe 11707 can detect *E. coli* DNA without amplification by a process of the invention.

Interrogation oligonucleotide:

11707 5' AGTGACTGGGG 3' SEQ ID NO:62

Example 19: <u>Digestion of PhiX 174 *Hin*F1 Fragments</u>

Polynucleotides encountered in nature are often double stranded. The DNA fragments generated by digestion of PhiX 174 DNA using endonuclease *Hin*FI are double-stranded DNA fragments of various sizes. In order to test whether double stranded DNA could be detected, PhiX 174 DNA was directly used as a target nucleic acid substrate or digested with nucleases to produce nucleotides that could be converted to nucleoside triphosphates as in previous examples.

The following conditions were used to digest DNA fragments from bacteriophage PhiX 174. The following materials were placed in three 1.5 mL polypropylene tubes: 50 μL of PhiX 174 *Hin*FI fragments (Promega G175A, Lot #773603); 40 μL 5 mM $MgSO_4$; 5 μL Exo III buffer (10X) (Promega E577B, 4853216), and 5 μL Nanopure water. Fifty microliters TE buffer and 40 μL 5 mM $MgSO_4$; 5 μL ExoIII buffer (10X) and 5 μL Nanopure water were added to one sample. Two of the samples containing PhiX 174 DNA were further treated with 2 μL Exo III (Promega M181A, 5512708) and the tubes placed in a 37°C water

bath for 60 minutes. ExoIII was also added to the sample without DNA and the sample incubated at 37°C 60 minutes.

At this time, 800 µL Nanopure water and 100 µL (10X) S1 Nuclease Buffer (Promega, M577A, Lot #6748605) were added to all samples. Three microliters S1 nuclease (Promega, E576B, Lot #789881) were then added to all samples. All samples were incubated at 37°C for 30 minutes.

Two hundred microliters from each of the three tubes containing DNA were diluted with 300 µL 1X TE Buffer and the absorbance read at 260 nm using a Beckman DU 650 spectrophotometer. The readings recorded were: tube one (no nuclease addition), 0.3073; tube two (treatment with Exo III), 0.5495; tube three (treatment with Exo III and S1), 0.5190. The increased absorbance values of the tubes treated with nuclease indicate that the polymer was digested. These digests were subsequently used in other studies (see Example 22, below).

Example 20: <u>Self-annealing Interrogation Probe</u>

This example illustrates use of a different type of oligonucleotide probe that is used to form a hairpin structure in the interrogation technology of this invention. This study demonstrates a method for eliminating the need for adding a probe specific to the interrogation site to the interrogation reaction.

Here, the oligonucleotide probe anneals to the target strand downstream of (3' to) the

interrogation position in the target strand. The oligonucleotide has at its 5' end an unannealed region of nucleotides followed by about 5 to about 20 nucleotides that are identical to the interrogation region on the target strand. The annealed 3' end of the oligonucleotide is then extended through the interrogation position of the target strand creating what is referred to as extended probe. The hybrid is denatured and a hairpin structure formed between the extended probe strand and the 5' end of the oligonucleotide probe. This region is then assayed in a standard interrogation reaction to determine if a mismatch is present or not.

Four probes were designed to represent different types of hairpin formations that an extended probe strands may assume. These probes are 10207 (SEQ ID NO:63), 10208 (SEQ ID NO:64), 10209 (SEQ ID NO:65), and 10212 (SEQ ID NO:66).

These probes are predicted to form the following self-hybridized secondary structures when allowed to self-anneal:

SEQ ID NO:63

```
10207     5' A-T-G-A-A-C-G-T-A-C-G-T-C-G-G
          3' T-A-C-T-T-G-C-A              |
                            \  C-G-A-G-T-A
```

SEQ ID NO:64

```
10208      5' G
                \
                 T-G-A-A-C-G-T-A-C-G-T-C-G-G
                  A-C-T-T-G-C-A             |
           3' T/               \C-G-A-G-T-A
```

SEQ ID NO:65

```
10209            A
                / \
           5' A-T   A-A-C-G-T-A-C-G-T-C-G-G
           3' T-A   T-T-G-C-A             |
                \C/          \C-G-A-G-T-A
```

SEQ ID NO:66

```
10212      5' A-T-A-A-A-C-G-T-A-C-G-T-C-G-G
                          3' G-C-A        /
                                  \C-G-A-G-T-A
```

A 5 μL (5 μg) aliquot of each of the four probes was diluted to 100 μL with nanopure water. They were then sequentially diluted 1:10 to a final dilution factor of 1:100,000. Twenty microliters of the diluted probes were heated, in separate tubes, at 95°C for 3 minutes and cooled to room temperature for 10 minutes to permit self-annealing. Twenty microliters of Master Mix, as described in Example 1, were then added to each tube and the tubes were incubated at 37°C for 15 minutes. Ten microliters of the solutions were added to 100 μL of L/L reagent (Promega, F202A) and relative light units measured immediately with a Turner® TD20/20 luminometer. The no-probe control resulted in 57.24 relative light

units and the remaining probe results are reported below in relative light units (rlu).

Log dilution	Probe 10207	10208	10209	10212
-5	44.89	56.22	57.57	57.80
-4	85.21	64.56	58.26	63.15
-3	297.7	70.53	79.12	82.65
-2	970.5	108.4	80.06	106.7

Probe 10207 worked as an efficient target for interrogation as expected, with probe 10208 providing the anticipated negative results. Probe 10212 has only a three base match so it may be un-extended, thus resulting in the low values. Probe 10209 likely has the 3' terminal nucleotide unannealed when the hairpin forms due to the mismatch at the third nucleotide in from the 3' end. Such an unannealed 3' terminal nucleotide would account for the low rlu values.

10207 5′ ATGAACGTACGTCGGATGAGCACGTTCAT 3′
SEQ ID NO:63

10208 5′ GTGAACGTACGTCGGATGAGCACGTTCAT 3′
SEQ ID NO:64

10209 5′ ATAAACGTACGTCGGATGAGCACGTTCAT 3′
SEQ ID NO:65

10212 5' ATAAACGTACGTCGGATGAGCACG 3'

SEQ ID NO:66

Example 21: Interrogation with a Self-Annealing Primer

This example and Fig. 2 illustrate use of a different type of oligonucleotide probe, a "REAPER™" probe in a process of this invention. This example demonstrates a method for eliminating the need for adding a probe specific to the interrogation site to the interrogation reaction.

Here, the oligonucleotide first probe (SEQ ID NO:68), at its 3'-end, anneals to the target strand (SEQ ID NO:67) at a position downstream of (3' to) the interrogation position in the target strand (Fig. 2A). The probe has at its 5'-end an unannealed region of nucleotides including about 5 to about 20 nucleotides that are identical to a region on the target strand including the interrogation position. This region of identity is present in the same orientation on both the target and the probe strands.

The annealed 3'-end of the probe is then extended through the interrogation position of the target strand forming what is referred to as a first extended probe and an extended first hybrid as is illustrated in Fig.2B (SEQ ID NO:69). The extended first hybrid is denatured and a second probe (SEQ ID NO:70) is annealed to the first extended probe to form a second hybrid. This second probe is complementary to the first extended probe strand at a

region downstream of the interrogation position on the first extended probe strand (Fig. 2C).

The second probe is then extended and a second extended hybrid is formed as illustrated in Fig. 2D. The second extended hybrid is comprised of the first extended probe and second extended probe (SEQ ID NO:71).

The strands of the second extended hybrid are denatured and permitted to renature to form a hairpin structure. Upon hairpin formation, the first extended probe forms a hairpin structure that has a 3'-overhang, whereas the second extended probe forms a hairpin structure that contains a 5'-overhang that provides a substrate for depolymerization. The second extended probe strand is then depolymerized and the analytical output obtained as described elsewhere herein. The analytical output determines the presence or absence of the original target strand or of a particular base in the original target strand as is also discussed elsewhere herein.

SEQ ID NO:67 oligonucleotide is diluted to 1 mg/mL in water. SEQ ID NO:70 oligonucleotide is diluted to 1 mg/mL in water. One microliter of each solution is combined with 18 μL water. The solution is heated to 95°C for 5 minutes then is cooled at room temperature for 10 minutes to permit oligonucleotides of SEQ ID NOs:67 and 70 to anneal.

To this solution are added dNTP mixture to a final concentration of 0.25 mM for each dNTP, 10X Klenow buffer to a final concentration of 1X, and 5 U of Klenow enzyme. The tube with these components is

incubated at 37°C for 30 minutes. The extended first hybrid DNA so formed (containing SEQ ID NO: 69) is purified (Qiagen, Mermaid system) and eluted into 50 µl of water.

To this solution of the purified extended first hybrid is added 1 µl SEQ ID NO: 70 oligonucleotide (1 mg/mL) as second probe. The solution is then heated to 95°C for 5 minutes and is cooled at room temperature to permit 69 and 70 to anneal as illustrated in Fig. 2C to form the second hybrid. To this solution are added a dNTP mixture to a final concentration of 0.25 mM for each dNTP, 10X Klenow buffer to a final concentration of 1X, and 5 U of Klenow enzyme. The tube with these components is incubated at 37°C for 30 minutes to form a second extended hybrid that contains a second extended probe (oligonucleotide SEQ ID NO: 71).

The SEQ ID NO: 71/69 second extended hybrid DNA (Fig. 2D) formed is purified (Qiagen, Mermaid system) to separate the extended hybrid from the unreacted dNTPs and eluted into 50 µl water. (Alternatively, the original 68 oligo is biotinylated at it's 5'-end and this biotin is then also present in strand of SEQ ID NO: 69. This biotinylated strand 69 is then denatured from strand 71 and removed from the solution with streptavidin coated paramagnetic particles according to the manufacturer's instructions (Promega, Z5481) and the 71 hairpin structure is allowed to form as below).

This hybrid solution is then heated to 95°C for 5 minutes diluted to 100 µl with water and is cooled on ice for 10 minutes to permit hairpin structure formation.

The following master mix is assembled and mixed.

Component	Amount
10X DNA Pol Buffer (Promega, M195A)	200 µL
Klenow exo- (1 U/µL) (Promega M218B)	12.5 µL
40 mM Sodium Pyrophosphate (Promega C350B)	25 µL
NDPK (1 U/µL)	10 µL
10uM ADP (Sigma A5285)	20 µL
Water	732.5 µL

Twenty microliters of this master mix are added to 20 µL of the above hairpin-containing solutions after cooling, and the resulting mixtures are heated at 37°C for 15 minutes. After this incubation, duplicate 4 µL samples of the solution are removed, added to 100 µL of L/L Reagent (Promega, F202A) and the light produced by the reaction is measured immediately using a Turner® TD20/20 luminometer. A positive analytical output at levels over background (no enzyme) indicates that a matched base was present at the 3'-terminus of the hairpin structure and this further indicates the presence of the target strand, and for this particular example,

it also indicates the presence of a G base at the interrogation position of the target.

5' CCCGGAGAGACCTCCTTAAGGGGCCATATTATTTCGTCGATTCCAGTGTT GGCCAAACGGAT 3' SEQ ID NO: 67

5' GGGGCCATATTATTTCGCCGTTTGGCCAACACTGGAATCGA 3' SEQ ID NO: 68

5' GGGGCCATATTATTTCGCCGTTTGGCCAACACTGGAATCGACGAAATAAT ATGGCCCCTTAAGGAGGTCTCTCCGGG 3' SEQ ID NO: 69

5' CCCGGAGAGACCTCCT 3' SEQ ID NO: 70

5' CCCGGAGAGACCTCCTTAAGGGGCCATATTATTTCGTCGATTCCAGTGTT GGCCAAACGGCGAAATAATATGGCCCC 3' SEQ ID NO: 71

Example 22: Detection of PhiX 174 *Hin*F1 Fragments Using Nucleases, PRPP Synthetase, NDPK

This example demonstrates the detection of DNA by digestion of the polymer to nucleoside monophosphates using nucleases, transformation of the nucleoside monophosphates to nucleoside triphosphates using PRPP Synthetase and PRPP along with transformation of ADP to ATP using the nucleoside triphosphates generated by the action of PRPP Synthetase, and detection of the ATP using luciferase. A sample of deoxynucleotide (Poly (dA)) was prepared as described in Example 19. Different

amounts of deoxynucleotide were used in the reactions as presented in Table 30.

The following additions were made to each reaction: 2 μL PRPP, 2 μL PRPP Synthetase, and 20 μL PRPP Synthetase buffer. The reactions proceeded at 37°C for 28 minutes, at which time the reactions were transferred to 100 μl LAR Buffer containing 2 μL ADP and 2 μL NDPK. This second reaction was permitted to proceed at room temperature for 20 minutes. The amount of ATP produced was measured by the addition of 10 ng of luciferase followed by measuring light output with a luminometer. The data are presented in table below. These data show that this combination of enzymes permitted detection of DNA.

Reaction	Nucleotide	Amount In Reaction	Light Units
1	dAMP	200 ng, 600 pmoles	1018
2	dAMP	20 ng, 60 pmoles	636
3	dAMP	2 ng, 6 pmoles	178
4	dAMP	200 pg, 600 fmoles	83
5	none	zero ng	69
6	PhiX 174 only	100 ng (= 300 pmoles dNMP; about 75 pmoles dAMP)	46
7	PhiX 174 + ExoIII	100 ng	472
8	PhiX 174 + Exo + S1	100 ng	448
9	No DNA + Exo + S1	zero ng	55

Example 23: Comparison of Thermophilic DNA Polymerases in a One-Step 70°C Interrogation Reaction

In this example, four different thermophilic DNA polymerases were used along with the thermophilic NDPK from *Pfu* in an interrogation reaction. The polymerases used were Taq (Promega, M166F), *Pfu* (*Pyrococcus furiosus* strain Vc1 DSM3638, Promega, M774A), Tvu (*Thermoactinomyces vulgaris*, purified at Promega), and Ath (*Anaeocellum thermophilum*, purified at Promega).

Cytomegalovirus (CMV) synthetic targets were generated by combining wild type oligonucleotide primers 9162 (SEQ ID NO:72) and 9165 (SEQ ID NO:73) or mutant oligonucleotide primers 9163 (SEQ ID NO:74) and 9166 (SEQ ID NO:75). The interrogation oligonucleotides used were wild type sequence 9211 (SEQ ID NO:35) and mutant sequence 9212 (SEQ ID NO:36).

Five nanograms of either the wild type or the mutant target (2.5 ng each of 9162 and 9165 for wild type or 9163 and 9166 for mutant) were combined with 1 μg of either the wild type probe, the mutant probe, or no probe, and water to a final volume of 20 μL. The solutions were heated for 5 minutes at 95°C then cooled for 10 minutes at room temperature. Twenty microliters of 2X master mix were then added to each solution, and each was further incubated at 70°C for 10 minutes. Four microliters of each solution were added to 100 μL of L/L Reagent (Promega F202A) and the relative light units (rlu) measured on

a Turner® TD20/20 luminometer. The various combinations of target and probe assayed and their average resulting rlu values, corrected for background values, from duplicate solutions are listed below.

2X Master Mix:

100 µL	10X Thermophilic DNA polymerase buffer (Promega, M190A)
100 µL	15 mM $MgCl_2$ (Promega, A351B)
25 µL	40 mM NaPPi (Promega, E350B)
10 µL	10 µM ADP (Sigma, A-5285)
5 µL	Thermophilic polymerase (1 U enzyme/reaction)
5 µl	*Pfu* NDPK (0.5 U/µL) (see Example 25 for enzyme purification; 0.1 U/rxn))
275 µL	water

Polymerase	Target	Probe	rlu	match:mismatch ratio
Taq	wild type	wild type	129	128:1
	wild type	mutant	-2	
	mutant	mutant	62	95:1
	mutant	wild type	0.65	
Pfu	wild type	wild type	121	20:1
	wild type	mutant	6	
	mutant	mutant	34	1:2
	mutant	wild type	54	
Tvu	wild type	wild type	898	89:1
	wild type	mutant	10	
	mutant	mutant	1075	66:1

	mutant	wild type	16	
Ath	wild type	wild type	327	327:1
	wild type	mutant	0	
	mutant	mutant	244	136:1
	mutant	wild type	1.8	

9162 5' CGTGTATGCCACTTTGATATTACACCCATGAACGTG CTCATCGACGTCAACCCGCACAACGAGCT 3' SEQ ID NO:72

9165 5' CGTTGTGCGGGTTCACGTCGATGAGCACGTTCATGG GTGTAATATCAAAGTGGCATACACGAGCT 3' SEQ ID NO:73

9163 5'CGTGTATGCCACTTTGATATTACACCCGTGAACGTG CTCATCGACGTCAACCCGCACAACGAGCT 3' SEQ ID NO:74

9166 5'CGTTGTGCGGGTTCACGTCGATGAGCACGTTCACGG GTGTAATATCAAAGTGGCATACACGAGCT 3' SEQ ID NO:75

9211 5'CACTTTGATATTACACCCATG 3' (wild type primer) SEQ ID NO:35

9212 5'CACTTTGATATTACACCCGTG 3' (mutant primer) SEQ ID NO:36

From the foregoing, it will be observed that numerous modifications and variations can be effected without departing from the true spirit and scope of the present invention. It is to be understood that no limitation with respect to the specific examples

presented is intended or should be inferred. The disclosure is intended to cover by the appended claims modifications as fall within the scope of the claims.